Death Receptor Regulation and Celecoxib-Induced Apoptosis in Human Lung Cancer Cells

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Background: **Celecoxib, a cyclooxygenase 2 inhibitor, has chemopreventive and therapeutic activities toward lung cancer and other epithelial malignancies. Celecoxib can induce apoptosis in various cancer cell lines through a mechanism that is independent of its cyclooxygenase 2 inhibitory activity but is otherwise largely uncharacterized. We investigated the mechanism of celecoxib-induced apoptosis further.** ***Methods:*** **All experiments were conducted in human non-small-cell lung carcinoma (NSCLC) cell lines; results in celecoxib-treated and untreated cells were compared. Cell survival was assessed with a sulforhodamine B assay. Apoptosis was assessed by DNA fragmentation with an enzyme-linked immunosorbent assay, by terminal deoxynucleotidyltransferase-mediated dUTP nick-end-labeling (TUNEL) assay, and by western blot analysis of caspase activation. Death receptor gene and protein expression was detected by northern and western blot analysis, respectively. Gene silencing was achieved with small interfering RNA (siRNA) technology.** ***Results:*** **Celecoxib treatment decreased cell survival, activated caspase cascades, and increased DNA fragmentation, all of which were abrogated when caspase 8 expression was silenced with caspase 8 siRNA. Celecoxib treatment induced the expression of death receptors, particularly that of DR5. Overexpression of a dominant negative Fas-associated death domain mutant, but not of BCL2, reduced the level of celecoxib-induced apoptosis, and silencing of DR5 expression by DR5 siRNA suppressed celecoxib-induced caspase 8 activation and apoptosis. Combination treatment with celecoxib and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced additional apoptosis. For example, survival of A549 cells was decreased with 50 μM celecoxib alone by 38.7% (95% confidence interval [CI] = 35.2% to 42.2%), with TRAIL alone by 29.3% (95% CI = 25.1% to 33.6%), but with their combination by 77.5% (95% CI = 74.5% to 79.5%), a greater than additive effect.** ***Conclusion:*** **Celecoxib appears to induce apoptosis in human NSCLC through the extrinsic death receptor pathway. [J Natl Cancer Inst 2004;96:1769–80]**

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Celecoxib was the first cyclooxygenase 2–selective nonsteroidal anti-inflammatory drug (NSAID) approved for the treatment of adult arthritis. Celecoxib exerts potent chemopreventive activity in chemical carcinogen–induced colon, bladder, and breast carcinogenesis *(1–4)* and UV-induced skin carcinogenesis *(5,6).* Celecoxib also effectively inhibits the growth of colon and breast cancer xenograft tumors in nude mice *(7–9)* and is a clinically effective chemoprevention agent for colon cancer *(10).* The U.S. Food and Drug Administration (FDA) has approved the use of celecoxib for the adjuvant treatment of familial adenomatous polyposis, an inherited syndrome that predisposes individuals to colon cancer. Currently, celecoxib is being tested in clinical trials for its chemopreventive or therapeutic activity against various cancers, including lung cancer, as a single agent or in combination with other agents.

Although celecoxib is a cyclooxygenase 2 inhibitor, it has been found to have antitumor activity in tumor cells and tissues that lack the cyclooxygenase 2 enzyme *(7–9,11,12).* Therefore, celecoxib appears to exert its chemopreventive and therapeutic activity through a mechanism that is independent of its cyclooxygenase 2 inhibitory activity. Celecoxib induces apoptosis in various cell types *(7,11–18),* and this activity may account for its chemopreventive and therapeutic activity *(19,20).* However, the mechanisms by which celecoxib induces apoptosis remain largely uncharacterized, although celecoxib-induced apoptosis appears to be associated with inactivation of the protein kinase Akt in prostate, colon, and liver cancer cells *(11,14,21)* and with inhibition of endoplasmic reticulum Ca^{2+}-ATPases in prostate cancer cells *(22).* Although celecoxib induces apoptosis when BCL2 is overexpressed *(11,17),* Jendrossek et al. *(17)* reported that celecoxib induced mitochondria-mediated apoptosis independent of the death receptor pathway in Jurkat T cells.

There are two major apoptotic signaling pathways, the intrinsic mitochondria-mediated pathway and the extrinsic death receptor–induced pathway, and cross-talk between these pathways is mediated by the truncation of the proapoptotic protein Bid *(23).* Steps in the intrinsic pathway include cytochrome *c* release from mitochondria, caspase 9 activation, and then activation of effector caspases, including caspase 3. Steps in the extrinsic pathway include the Fas-associated death domain (FADD)-dependent recruitment and activation of caspase 8 and/or caspase 10, triggered by the binding of a death receptor ligand to its death receptor, and then activation of the same effector caspases involved in the intrinsic pathway. The antiapoptotic molecules BCL2 and BCL-XL inhibit the intrinsic pathway by preventing cytochrome *c* release from mitochondria. Many chemicals or small molecules induce apoptosis through the intrinsic pathway *(24),* but other small molecules, including NSAIDs, induce apoptosis through the extrinsic pathway by increasing the expression of death ligands *(25)* or death receptors *(26,27).*

The importance of death receptors in NSAID-induced apoptosis has been described previously. Han et al. *(27)* reported that the NSAIDs indomethacin and sulindac activate caspase 8 and apoptosis by a FADD-dependent mechanism in Jurkat T cells. Huang et al. *(26)* found that sulindac induces DR5 expression in human prostate and colon cancer cells and that DR5 expression then contributes to sulindac-induced apoptosis.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL, also called APO-2L) is a member of the tumor necrosis factor family that appears to induce apoptosis in a wide variety of transformed cells but does not appear to induce apoptosis in normal cells *(28,29).* TRAIL appears to be a tumor-selective, apoptosis-inducing cytokine and may be a potential agent for cancer treatment. TRAIL induces apoptosis by binding to one of the two death domain–containing receptors—DR4 (also called TRAIL receptor 1) and DR5 (also called TRAIL receptor 2). TRAIL can also bind to the decoy receptors DcR1 (also called TRAIL-R3) and DcR2 (also called TRAIL-R4), but these receptors contain either no cytoplasmic death domain or a truncated death domain and so cannot transmit a signal to induce apoptosis. Because DcR1 and DcR2 can compete with DR4 and DR5 for TRAIL binding, they act to inhibit TRAIL-induced apoptosis *(28,29).* TRAIL induces apoptosis by binding to its cognate death receptors (i.e., DR4 or DR5), which then recruit caspase 8 via the FADD. Activated caspase 8 then directly activates caspases 3, 6, and 7 or activates the intrinsic mitochondria-mediated pathway through caspase 8–mediated Bid cleavage, which indirectly activates caspases 3, 6, and 7 *(28,29).*

Both DR5 and DR4 are target genes of p53 *(30–33),* but they can also be regulated through p53-independent mechanisms *(34–36).* Therefore, drug-induced expression of DR5 and DR4 can be either p53-dependent or p53-independent. It has been reported that the NSAID sulindac induces DR5 expression in human colon and prostate cancer cells in an apparently p53-independent manner *(26).* In general, agents that increase the expression of TRAIL death receptors are able to enhance TRAIL-induced apoptosis in human cancer cells *(29).*

In this study, we examined the mechanism of celecoxib-induced apoptosis in four human non-small-cell lung cancer (NSCLC) cell lines. We were particularly interested in the roles of caspase 8 activation, death receptor DR5 and DR4 expression, and TRAIL in this mechanism.

Materials and Methods

Reagents

Celecoxib was purchased from LKT Laboratories (St. Paul, MN). The cyclooxygenase 2 inhibitor NS398 was purchased from Biomol (Plymouth Meeting, PA). DUP697 (a cyclooxygenase 2 inhibitor), SC-58125 (a cyclooxygenase 2 inhibitor), sulindac sulfide (a cyclooxygenase 1 and 2 inhibitor), sulindac sulfone (a sulindac sulfide metabolite without cyclooxygenase inhibitor activity), and SC-560 (a cyclooxygenase 1 inhibitor) were purchased from Cayman Chemical (Ann Arbor, MI). Each of these compounds was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 m*M* and stored at −80 °C. Stock solutions were diluted to the appropriate concentrations with growth medium immediately before use. Other agents were purchased from Sigma Chemical (St. Louis, MO).

Cell Lines and Cell Culture

Human NSCLC cell lines H460, A549, H358, and H1792 were purchased from the American Type Culture Collection (Manassas, VA). Among these cell lines, A549 and H358 cells express high levels of cyclooxygenase 2, whereas H460 and H1792 cells express very low levels of cyclooxygenase 2 (data not shown). H460 cells stably transfected with control vector (H460/V) and with BCL2 expression vector (H460/Bcl2-6) were described previously *(37).* These cell lines were grown in mono-

layer culture in a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F12 medium supplemented with 5% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

Generation of Stable Transfectants That Overexpress a Dominant Negative FADD Mutant

NSCLC H460 cells were transfected with the control pcDNA3 empty vector or with the vector containing the dominant negative mutant FADDm, termed pcDNA-FADD-DN *(38),* by use of FuGene 6 (Roche Molecular Biochemicals, Indianapolis, IN), in accordance with the manufacturer's instructions. After selection in culture medium containing G418 (500 μg/mL; Gibco-BRL, Rockville, MD) for 2 weeks, individual G418-resistant clones were isolated with cloning cylinders and cultured to obtain adequate numbers of cells for other experiments. Expression of the dominant negative mutant FADDm was confirmed by western blot analysis with mouse anti-FADD monoclonal antibodies (Upstate Biotechnology, Placid Lake, NY). Cells transfected with the empty vector (that expressed no FADDm) were designated H460/V1, and cells transfected with the vector containing FADDm that expressed FADDm were designated H460/Fm6 and H460/Fm16.

Cell Viability and Death Assays

Cells were cultured in 96-well cell culture plates (viability assay) or in 10-cm diameter dishes (death assay), treated on the second day with the agents indicated, and then subjected to an assay. For the cell viability assay, the viable cell number was estimated by use of the sulforhodamine B assay, as previously described *(39).* For the cell death assay, the number of cells floating in the medium were directly counted with a hemacytometer.

Apoptosis Assays

Cells were cultured in 96-well cell culture plates or in 10-cm diameter dishes and treated with test agents on the second day as indicated, and then apoptosis was assessed by use of three assays. The first assay used the presence of cytoplasmic histone-associated DNA fragments (mononucleosome and oligonucleosomes) to identify apoptotic cells by use of an enzyme-linked immunosorbent assay (Cell Death Detection $ELISA_{Plus}$ kit; Roche Molecular Biochemicals), according to the manufacturer's instructions. The second assay for apoptotic cells was the terminal deoxynucleotidyltransferase-mediated dUTP nick-end-labeling (TUNEL) assay. For this assay, cells were cultured on 10-cm diameter dishes for 1 day, then treated with agents as indicated, and harvested by trypsinization. Cells were fixed with 1% paraformaldehyde, and cytoplasmic DNA fragments with 3′-hydroxyl ends were detected with an APO-Direct TUNEL kit (Phoenix Flow Systems, San Diego, CA) by following the manufacturer's protocol. The third assay for apoptosis measured the level of activated caspase. In this assay, cells were harvested, whole cell protein lysates were prepared, and activation of caspases was assessed in the lysates by western blot analysis as described below.

Western Blot Analysis

Preparation of whole cell protein lysates and western blot analysis were as described previously *(40).* Briefly, whole cell protein lysates (50 μg) were electrophoresed through 7.5%–12% denaturing polyacrylamide slab gels, and the protein bands were transferred to a Hybond enhanced chemiluminescence (ECL) membrane (Amersham, Arlington Heights, IL) by electroblotting. The blots were probed or re-probed with the appropriate primary antibodies, blots were incubated with the secondary antibodies, and then antibody binding was detected by the ECL system (Amersham), according to the manufacturer's protocol. Mouse anti-caspase 3 and anti-DR4 monoclonal antibodies and rabbit anti-DR5 polyclonal antibody were purchased from Imgenex (San Diego, CA). Rabbit anti-caspase 9, anti-caspase 8, anti-caspase 7, anti-caspase 6, anti-poly(ADP-ribose) polymerase (PARP), anti-lamin A/C, and anti-DNA fragmentation factor 45 (DFF45) polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-Bid polyclonal antibody was purchased from Trevigen (Gaithersburg, MD). Mouse anti-BCL2 monoclonal antibody was purchased from Santa Cruz Technology (Santa Cruz, CA). Rabbit anti-β-actin polyclonal antibody was purchased from Sigma. Secondary antibodies, goat anti-mouse immunoglobulin G (IgG)–horseradish peroxidase conjugates and anti-rabbit IgG–horseradish peroxidase conjugates, were purchased from Bio-Rad (Hercules, CA) and Pierce Biotechnology (Rockford, IL), respectively.

Northern Blot Analysis

Total cellular RNA was prepared and loaded (30 μg of RNA per lane) onto a formaldehyde-denatured agarose gel, and RNAs were separated by electrophoresis. Northern blot analysis was performed as described previously *(39).* The probes were human DR5 cDNA (obtained from Dr. W. S. El-Deiry, The University of Pennsylvania School of Medicine, Philadelphia, PA); human DR4 cDNA (purchased from Alexis Biochemicals, San Diego, CA); and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (purchased from Ambion, Austin, TX).

Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of DR5

Cells were cultured on 10-cm dishes in culture medium supplemented with 5% fetal bovine serum and treated with celecoxib, as indicated, on the second day. After a 24-hour incubation, the level of DR5 protein was measured in cells with an ELISA kit purchased from Biosource International (Camarillo, CA), by following the manufacturer's instructions.

Silencing of Gene Expression With Small Interfering RNA

Gene silencing by small interfering RNA (siRNA) technology uses a small double-stranded RNA (i.e., the siRNA) that triggers degradation of target mRNA. Gene silencing was achieved by transfecting cells with siRNAs by use of the RNAiFect transfection reagent (Qiagen, Valencia CA), following the manufacturer's instructions. High-purity control (i.e., non-silencing) siRNA oligonucleotides that target the sequence 5′-AATTCTCCGAACGTGTCACGT-3′ were purchased from Qiagen. This scrambled sequence does not match any human genome sequence. Caspase 8 and DR5 siRNA duplexes that target the sequences 5′-AACTACCAGAAAGGTATACCT-3′ and 5′-AAGACCCTTGTGCTCGTTGTC-3′, respectively, as described previously *(41,42),* were synthesized by Qiagen. To improve gene silencing, we transfected the same cells twice with the same siRNA with a 48-hour interval between the two trans-

fections. Twenty-four hours after the second transfection, cells were re-plated in fresh medium supplemented with 5% fetal bovine serum and treated on the second day with celecoxib, as indicated. Gene silencing effects were evaluated by western blot analysis.

Statistical Analysis

The statistical significance of differences in cell survival and apoptosis (i.e., DNA fragmentation) between two groups were analyzed with two-sided unpaired Student's *t* tests when the variances were equal or with Welch's corrected *t* test when the variances were not equal, by use of Graphpad InStat 3 software (GraphPad Software, San Diego, CA). Data were examined as suggested by the same software to verify that the assumptions for use of the *t* tests held. All means and 95% confidence intervals (CIs) from triplicate or four replicate samples were calculated with Microsoft Excel software, version 5.0 (Microsoft, Seattle, WA). Results were considered to be statistically significant at $P<.01$ to account for multiple comparisons. All statistical tests were two-sided.

Results

Celecoxib, Caspase 8, and Apoptosis in Human NSCLC Cell Lines

To determine the optimal celecoxib concentration and incubation time for our experiments, we treated the following four NSCLC cell lines H358, A549, H1792, and H460 with celecoxib and examined cell survival. The survival of all four cell lines was reduced by celecoxib in a concentration-dependent manner (Fig. 1, A) when incubated for 24, 48, or 72 hours. The concentration of celecoxib that resulted in a 50% decrease in cell survival after a 48-hour incubation averaged about 30 μ*M* in these cell lines. A 48-hour incubation with celecoxib reduced cell survival more effectively than a 24-hour incubation, but a 72-hour incubation did not reduce cell survival further. To achieve biologically significant effects in the subsequent experiments, we used a celecoxib concentration of 50 μ*M* and an incubation time of not more than 48 hours.

We next examined whether celecoxib treatment activated caspase cascades in these cell lines by monitoring the cleavage

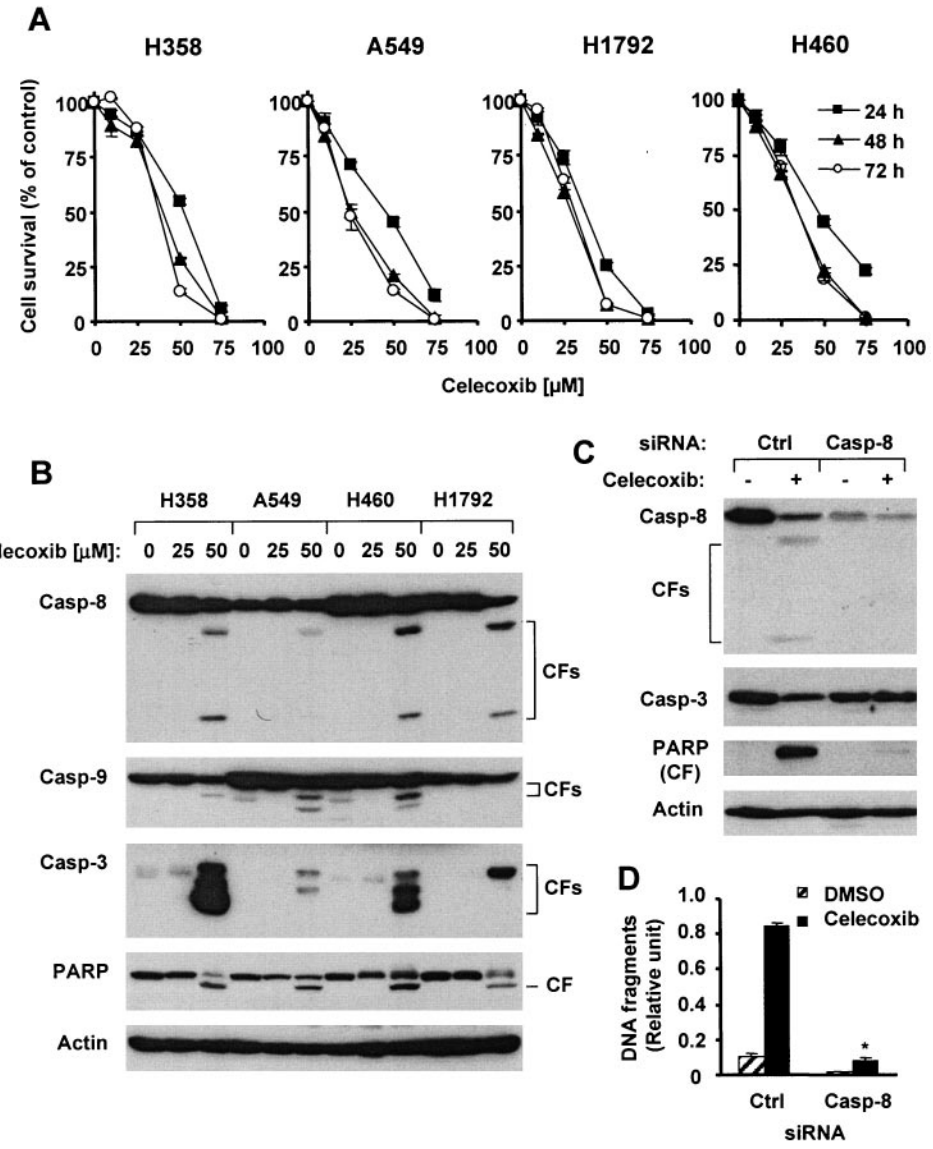

Fig. 1. Celecoxib and caspase 8-mediated apoptosis in human non-small-cell lung carcinoma (NSCLC) cell lines. **A**) Celecoxib and cell survival. NSCLC cell lines, as indicated, were cultured in 96-well cell culture plates and treated on the second day with celecoxib as indicated. After 24, 48, or 72 hours, cell numbers were estimated by use of the sulforhodamine B assay, as described *(39).* Cell survival is expressed as the percent of control (dimethyl sulfoxide [DMSO]-treated) cells (0 μ*M* celecoxib). Each point is the mean value from four identical wells. **Error bars** = 95% confidence intervals [CIs]. **B**) Celecoxib and caspase activation. NSCLC cell lines were treated with celecoxib, as indicated, for 31 hours, whole cell protein lysates were prepared, and cleavage of caspase 8 (Casp-8), caspase 9 (Casp-9), caspase 3 (Casp-3), and poly(ADP-ribose) polymerase (PARP) was detected by western blot analysis. (Cleaved forms of caspase 9 appeared with longer exposure in the H1792 cell line, as did the smaller cleaved form of caspase 8 in the A549 cell line; data not shown.) Actin expression was used as a loading control. CF = cleaved form. **C** and **D**) Silencing of caspase 8 expression and celecoxib-induced caspase activation and DNA fragmentation. H1792 cells were cultured in a 24-well cell culture plate and transfected with control (Ctrl) or caspase 8 small interfering RNA (siRNA) twice with a 48-hour interval between transfections. Forty hours after the second transfection, cells were treated with DMSO (0.05%) or 50 μ*M* celecoxib. Twenty-four hours later, caspase activation was assessed by western blot analysis (**C**), and DNA fragmentation was assessed with the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals) (**D**). Data are the mean value of three identical wells, and error bars are the upper 95% CIs. *, $P<.001$ compared with that of control siRNA-transfected cells treated with celecoxib. All statistical tests were two-sided.

of upstream and downstream components of caspase cascades, caspases 3, 8, and 9 and PARP. Addition of 50 μ*M* celecoxib induced cleavage of caspases 8 and 9, as well as cleavage of caspase 3 and its substrate PARP (Fig. 1, B). Thus, celecoxib appears to activate the caspase cascade in NSCLC cells.

To determine whether caspase 8 activation is involved in celecoxib-induced apoptosis, we transfected H1792 cells (which tolerate transfection reagents better than other cell lines evaluated and have good transfection efficiency) with caspase 8 siRNA to silence caspase 8 expression and then assessed whether apoptosis was altered by examining levels of cleaved and uncleaved caspases 8 and 3 and PARP, as well as levels of DNA fragmentation. We found that, in the absence of celecoxib treatment, the level of caspase 8 was much lower in cells transfected with caspase 8 siRNA than in cells transfected with control siRNA (Fig. 1, C). After treatment of control siRNA-transfected cells with 50 μ*M* celecoxib, levels of uncleaved forms of caspase 8 decreased as levels of cleaved forms of caspase 8 increased, the level of uncleaved caspase 3 was reduced, and levels of cleaved PARP and DNA fragments were elevated, all compared with levels in corresponding untreated cells (Fig. 1, C and D). After caspase 8 siRNA-transfected cells were treated with 50 μ*M* celecoxib, the level of uncleaved caspase 3 appeared unchanged, cleaved PARP was faintly detected, and DNA fragmentation was statistically significantly less than that in celecoxib-treated control siRNA–transfected cells (Fig. 1, C and D). Similar results were obtained with H460 cells (data not shown). Thus, celecoxib appeared to activate apoptosis in a caspase 8–dependent manner in human NSCLC cells.

Celecoxib and DR5 Expression

To determine whether celecoxib induces DR5 expression and whether p53 status alters the effect of celecoxib, we examined the effect of celecoxib on DR5 expression in three human NSCLC cell lines, one carrying wild-type p53 (A549), one carrying mutant p53 (H1792), and one that is p53-null(H358) *(43).* We treated all three lines with celecoxib and then assessed DR5 mRNA and protein expression. The level of DR5 mRNA increased in a time-dependent manner in all three cell lines, regardless of p53 status, for at least 24 hours after celecoxib treatment, compared with that in untreated cells (Fig. 2, A). The level of DR5 protein also increased in all three cell lines after a 24-hour incubation with celecoxib, compared with that in untreated cells, particularly when cells were treated with 50 μ*M* celecoxib (Fig. 2, B and C). Thus, celecoxib appears to induce DR5 expression in a p53-independent manner in human NSCLC cells.

To determine whether celecoxib increases DR5 mRNA expression at the transcriptional level, we used the transcriptional inhibitor actinomycin D and the translational inhibitor cycloheximide. DR5 mRNA was not detected in cells treated with actinomycin D or in cells treated with the combination of actinomycin D and celecoxib. Treatment of cells with cycloheximide alone increased the level of DR5 mRNA, compared with that in untreated control cells, and treatment with both cycloheximide and celecoxib increased the level of DR5 mRNA even more (Fig. 2, D). We next examined whether celecoxib altered the stability of the DR5 mRNA. After cells were treated with DMSO (vehicle control) or with celecoxib for 24 hours, actinomycin D was then added, and mRNAs were isolated from cells after 0, 1,

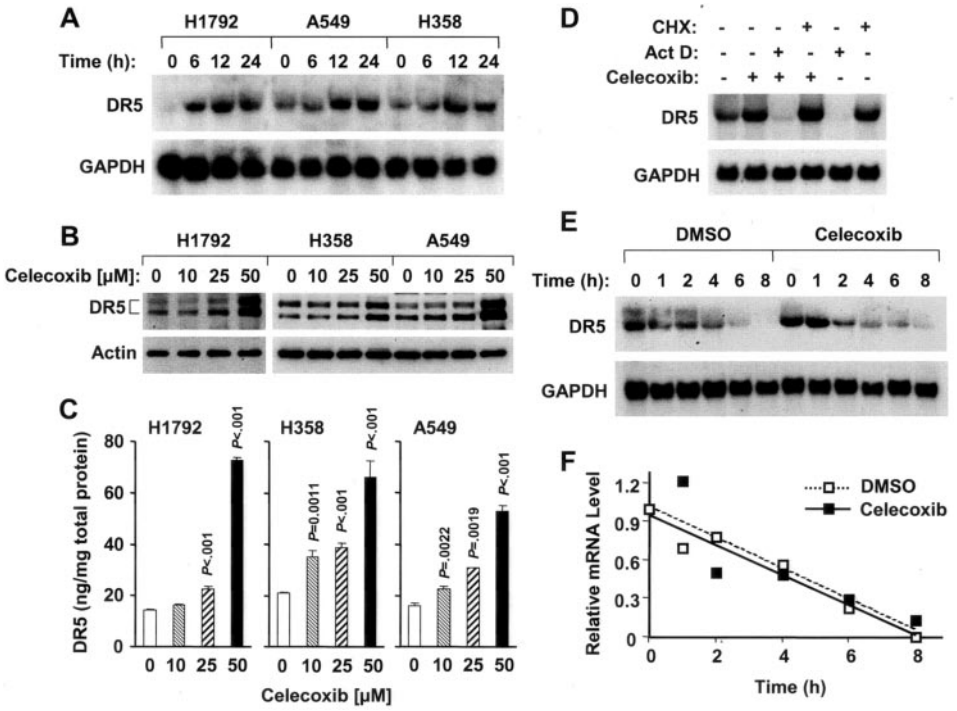

Fig. 2. Celecoxib and DR5 expression in human non-small-cell lung carcinoma (NSCLC) cells. **A–C**) Celecoxib and DR5 mRNA (**A**) and protein (**B** and **C**) expression. NSCLC cell lines were treated with 50 μ*M* celecoxib for the times indicated (**A**) or concentrations of celecoxib indicated for 24 hours (**B** and **C**). Total cellular RNA and whole cell protein lysates were then prepared, DR5 mRNA was detected by northern blot analysis (**A**), and DR5 protein was detected by western blot analysis (**B**) and by enzyme-linked immunosorbent assay (ELISA) (**C**). Data in **panel C** are the mean value of three identical treatments, and **error bars** are the upper 95% confidence intervals [CIs]. All *P* values were compared with control cells (0 μ*M* celecoxib) for all cells treated with the indicated concentrations of celecoxib compared with control cells. All statistical tests were two-sided. **D**) Celecoxib induction of DR5 expression. H358 cells were pretreated with the transcription inhibitor actinomycin D at 5 μg/mL or with the translational inhibitor cycloheximide (CHX) at 10 μg/mL for 30 minutes and then treated with the combination of actinomycin D (Act D) or cycloheximide and 50 μ*M* celecoxib for 24 hours. Total cellular RNA was then prepared and the expression of DR5 mRNA was detected bynorthern blot analysis. **E** and **F**) Celecoxib and the stability of DR5 mRNA in H358 cells. **E**) After a 24-hour treatment with 50 μ*M* celecoxib or dimethyl sulfoxide (DMSO), cells were exposed to actinomycin D at 5 μg/mL, and total cellular RNA was isolated and examined by northern blot analysis. **F**) Hybridization signals were quantitated with a PhosphorImager using ImageQuant software and were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are the relative level of mRNA (ratio of the value at time 0 of actinomycin D treatment) at the indicated times. The result reflects one gel scanning.

2, 4, 6, and 8 hours of incubation to assess the degradation rate of DR5 mRNA (Fig. 2, E and F). The rate of DR5 mRNA degradation in control (DMSO)-treated cells was similar to that in celecoxib-treated cells. Thus, celecoxib appeared to increase DR5 expression at the transcriptional level.

Celecoxib and the Expression of the Other TRAIL Receptors DR4 and the Decoy Receptors

To determine whether celecoxib also affects the expression of TRAIL receptors in addition to that of DR5, we treated cells with celecoxib for 24 hours and assessed the levels of DR5, DR4, DcR1, and DcR2 mRNAs. Celecoxib treatment increased the levels of DR4 and DR5 mRNAs in a concentration-dependent manner, particularly in H358 cells (Fig. 3, A) but did not change the levels of DcR1 and DcR2 mRNAs in all three cell lines tested. In H358 cells, celecoxib at concentrations from 5 μM through 50 μM increased the expression of DR4, whereas in A549 and H1792 cells, increased DR4 expression was detected even at celecoxib concentrations of 25 μM and 10 μM, respectively. Because celecoxib increased the DR4 mRNA level but did not change DcR1 and DcR2 mRNA levels, we also examined the effect of celecoxib on DR4 protein expression. Increased expression of DR4 protein was detected at 25 μM celecoxib in H358 cells and at 50 μM celecoxib in H1792 cells, but the expression of DR4 protein in A549 cells was not altered, even at 50 μM celecoxib (Fig. 3, B). Thus, we conclude that celecoxib also increased DR4 expression in some NSCLC cell lines.

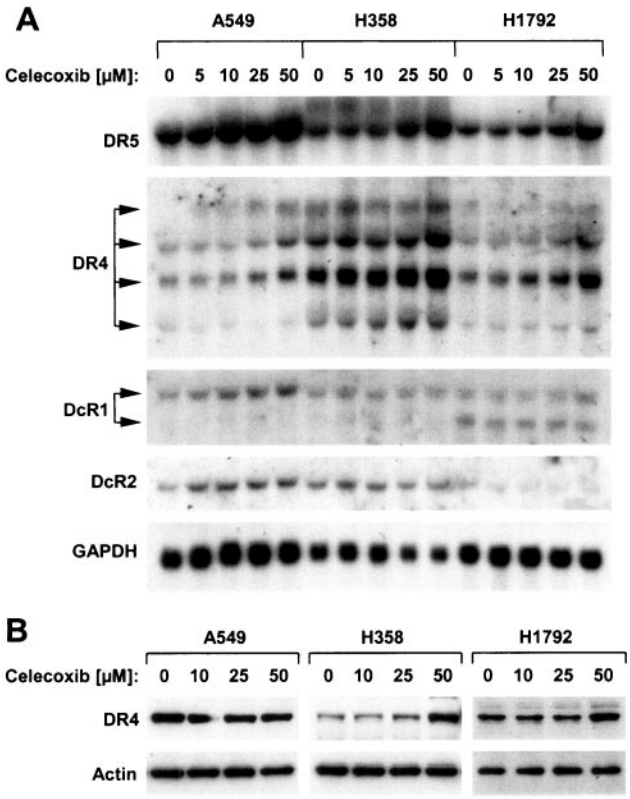

Fig. 3. Celecoxib and modulation of the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors (DR4, DR5, DcR1, and DcR2) in human non-small-cell lung carcinoma (NSCLC) cells. **A**) Celecoxib and the expression of TRAIL receptors. NSCLC cell lines as indicated were treated with celecoxib for 24 hours as indicated. Total cellular RNAs were prepared and subjected to northern blot analysis to detect TRAIL receptor expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a loading control. **B**) Celecoxib and DR4 protein expression. The indicated NSCLC cell lines were treated with celecoxib as indicated. After 24 hours, whole cell protein lysates were prepared, and DR4 protein was detected by western blot analysis. Actin expression level was used as a loading comparison.

NSAIDs, Induction of DR5 Expression, and Apoptosis

To determine whether other NSAIDs also modulate death receptor expression in human NSCLC cells, we treated H358 cells with celecoxib or another NSAID (NS398, DUP697, SC-58125, sulindac sulfide, sulindac sulfone, or SC-560; each at 50 μM) and then assessed death receptor protein expression and survival in these cells. Treatment with celecoxib, NS398, DUP697, or SC-58125 increased the expression of DR5 protein (Fig. 4, A), with celecoxib and DUP697 inducing the highest levels, and increased the expression of DR4 protein, with cele-

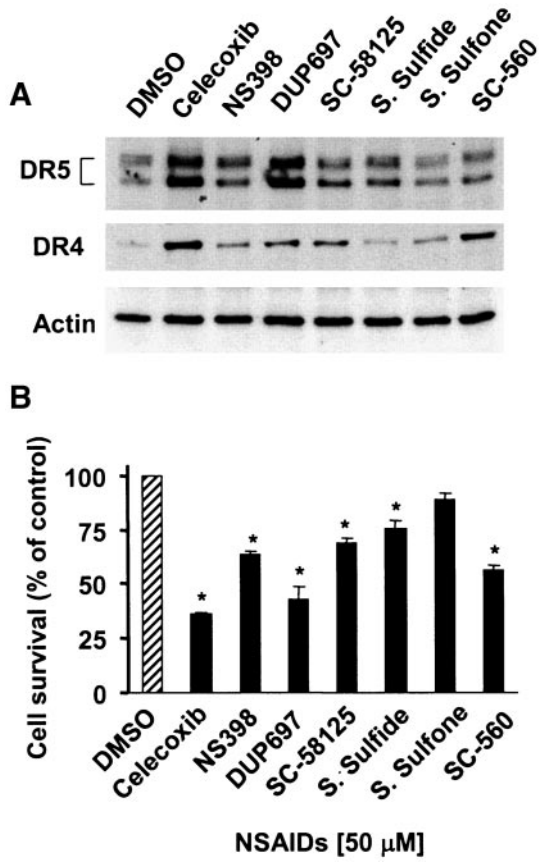

Fig. 4. Abilities of celecoxib and other nonsteroidal anti-inflammatory drugs (NSAIDs) to induce death receptor expression (**A**) and apoptosis (**B**). **A**) H358 cells were treated for 24 hours with dimethyl sulfoxide (DMSO), 50 μM celecoxib, or the indicated NSAIDs at 50 μM. Whole cell protein lysates were prepared, and DR4 or DR5 protein was detected with western blot analysis. Actin expression was used as a loading control. **B**) H358 cells were cultured in 96-well cell culture plates and treated with DMSO, 50 μM celecoxib, or other NSAIDs at 50 μM on the second day. After 48 hours, the cell number was estimated by use of the sulforhodamine B assay, as described *(39)*. Cell survival data are expressed as percent of control DMSO-treated cells and are the mean value of four identical wells. **Error bars** are the upper 95% confidence interval. *, $P<.001$ for survival cells treated with the indicated agent relative to control DMSO-treated cells. All statistical tests were two-sided. S. Sulfide = sulindac sulfide; S. Sulfone = sulindac sulfone.

coxib inducing the highest level. Although treatment with SC-560 did not increase DR5 expression, it did increase DR4 expression. Treatment with either sulindac sulfide or sulindac sulfone did not appear to induce DR4 expression, although treatment with sulindac sulfide increased the expression of DR5 slightly.

When the induction of DR4 protein expression was used to assess NSAID activity in H358 cells, the order from most active to least active was celecoxib, SC-560, DUP697, SC-58125, and NS398. When cell survival was used to assess NSAID activity in H358 cells, treatment with each NSAID decreased cell survival, with celecoxib and DUP697 having the highest activity and sulindac sulfone having the lowest activity (Fig. 4, B). The ability of each NSAID, except for SC-560, to decrease cell survival appeared to be associated with its ability to induce the expression of DR5 (Fig. 4). The ability of SC-560 to decrease cell survival may be related to its ability to induce DR4 expression. The inability of sulindac sulfone to increase the expression of DR5 and DR4 may account for its inability to decrease cell survival. Thus, the induction of death receptors, particularly DR5 induction, appeared to be important for NSAID-induced apoptosis, at least in human NSCLC cell lines.

BCL2 Overexpression and Resistance to Celecoxib-Induced Apoptosis

BCL2 is an antiapoptotic protein that inhibits the release of cytochrome *c* from mitochondria into the cytoplasm, thereby inhibiting intrinsic mitochondria-mediated apoptosis *(23,24)*. To determine whether BCL2 overexpression protects cells from celecoxib-induced apoptosis, we used H460 cells transfected with an empty control vector (H460/V) and H460 cells transfected with a vector containing the gene for BCL2 (H460/Bcl2-6). Treatment with increasing concentrations of celecoxib did not alter the expression of endogenous (i.e., genomic) or exogenous (i.e., transfected) BCL2 or of DR4 protein in either cell line (Fig. 5, B). Celecoxib did not differentially alter survival of either of the two cell lines (Fig. 5, A), and it increased the level of DR5 protein equally well in both cell lines (Fig. 5, B). Thus, overexpression of BCL2 did not appear to confer resistance to celecoxib.

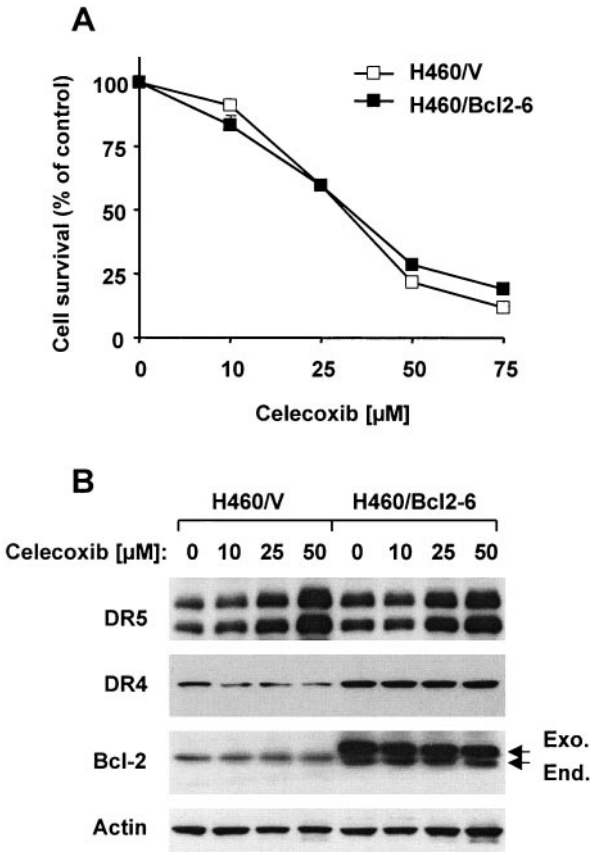

Fig. 5. BCL2 overexpression, celecoxib-induced apoptosis (**A**), and death receptor expression (**B**). **A**) Control vector-transfected H460/V and BCL2 vector-transfected H460/Bcl2–6 cells were cultured in 96-well plates and treated on the second day with celecoxib as indicated. After 3 days, cell number was estimated with the sulforhodamine B assay, as described *(39)*. Cell survival data are expressed as percent of control dimethyl sulfoxide-treated cells (0 μM celecoxib). Data are the mean value of four identical wells, and **error bars** are the 95% confidence intervals (some error bars are too small to be seen). **B**) H460/V and H460/Bcl2–6 cells were cultured in 10-cm dishes and treated with celecoxib as indicated for 24 hours. Whole cell protein lysates were then prepared, and DR5, DR4, and BCL2 expression levels were detected by western blot analysis. Actin expression was used as a loading control. Positions of exogenous (Exo.) and endogenous (End.) BCL2, DR5, and DR4 are indicated.

Death Receptor Expression and Celecoxib-Mediated Apoptosis

The binding of TRAIL to DR5 or DR4 recruits and activates caspase 8 via the adaptor molecule FADD, and activated caspase 8 induces apoptosis by activating caspase cascades *(28,29)*. Death receptor–induced apoptosis can be disrupted by use of a dominant negative FADD mutant (FADDm) that blocks the recruitment of caspase 8 *(38)*. To determine whether increased death receptor expression contributes to celecoxib-induced apoptosis, we transfected cells with a FADDm expression vector or with an empty control vector (Fig. 6, A), treated the transfectants with celecoxib, and assessed their survival. Celecoxib decreased cell survival better in cells transfected with the empty vector (H460/V1) than in the two lines transfected with FADDm (H460/Fm6 and H460/Fm16) (Fig. 6, B). After a 24-hour treatment with 50 μM celecoxib, survival of H460/V1 cells was 28.2% (95% CI = 28.0% to 29.6%) of that of untreated cells, survival of H460/Fm6 cells was 47.1% (95% CI = 45.0% to 49.2%) of that of untreated cells, and survival of H460/Fm16 was 44.5% (95% CI = 43.1% to 45.9%) of that of untreated cells, with the survival values of H460/Fm6 and H460/Fm16 being statistically significantly different from that of H460/V1 ($P<.001$).

In addition to measuring viable cells, we also measured dead cells by directly counting the number of floating cells in the medium. As shown in Fig. 6, C, the number of floating or dead cells was statistically significantly increased in cultures of H460/V1 cells but not in cultures of H460/Fm6 and H460/Fm16 cells after exposure to celecoxib. Thus, the overexpression of FADDm appeared to protect cells from celecoxib-induced cell death.

When we directly measured DNA fragmentation and caspase activation—hallmarks of apoptosis—we found that the differences in the response to celecoxib between empty vector–transfected and FADDm-transfected H460 cells were apparently larger than what we observed in the cell survival assay. After treatment with 50 μM celecoxib, the amount of DNA fragmentation was increased in H460/V1 cells but not in H460/Fm6 cells

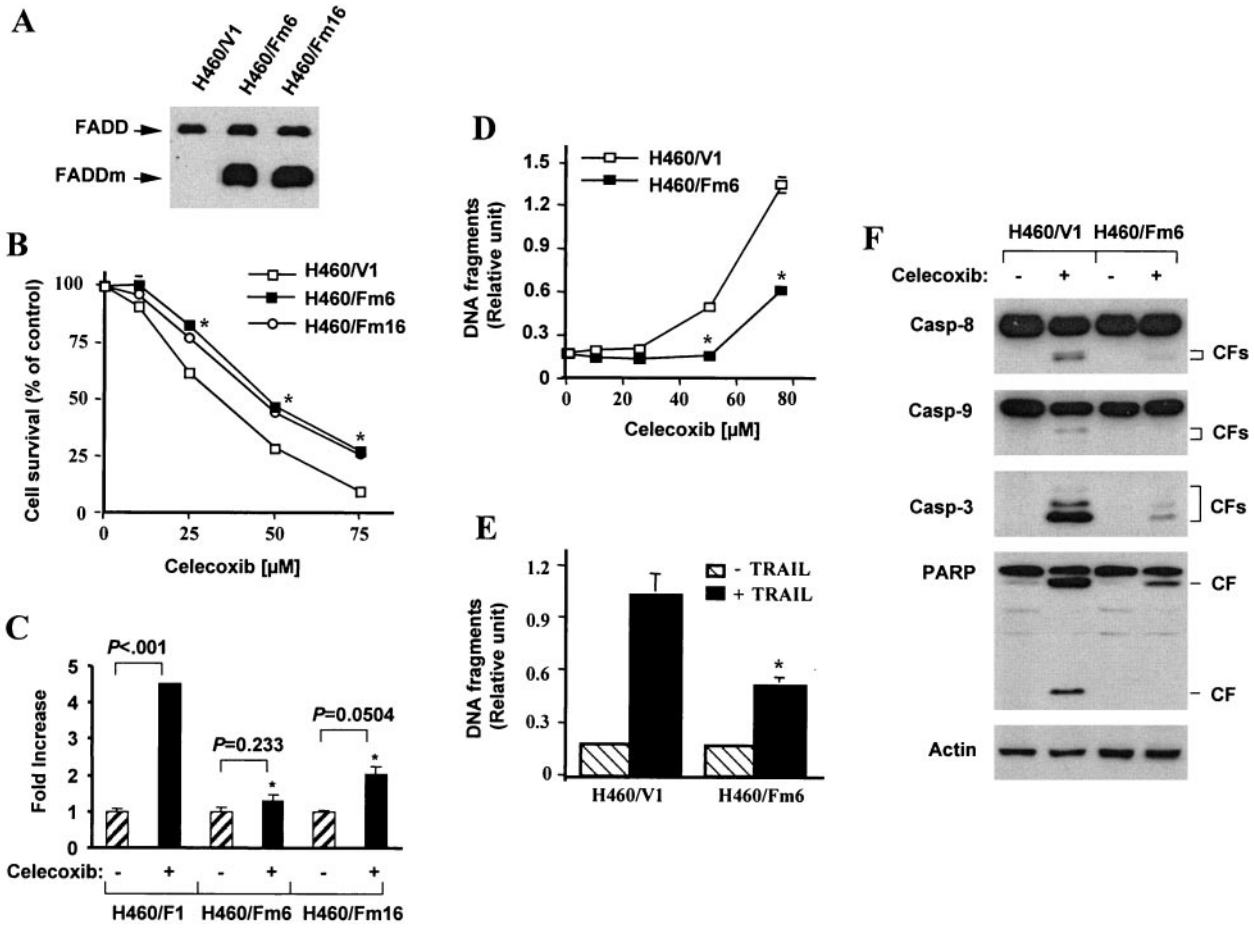

Fig. 6. Overexpression of a dominant negative Fas-associated death domain mutant (FADDm) and celecoxib-induced apoptosis. **A**) Detection of FADDm by western blot analysis. Whole cell protein lysates prepared from H460 transfectants as indicated were subjected to detection of endogenous FADD and FADDm by western blot analysis. **B**) Overexpression of FADDm and celecoxib-induced decrease of cell survival. H460 cells transfected with empty vector (H460/V1) or FADDm (H460/Fm6 and H460/Fm16) were cultured in 96-well plates and treated on the second day with celecoxib as indicated. After 24 hours, the cell number was estimated with the sulforhodamine B assay, as described *(39).* Cell survival data are expressed as percent of dimethyl sulfoxide-treated cells (0 μ*M* celecoxib). Data are the mean value of four identical wells, and **error bars** are the 95% confidence intervals. *, $P<.001$ compared with celecoxib-treated H460/V1 cells. **C**) Overexpression of FADDm and celecoxib-induced cell death. Cell lines, as indicated, were cultured in 10-cm diameter dishes and treated with 50 μ*M* celecoxib. After 24 hours, the floating or dead cells in the medium were counted directly with a hemacytometer. Data are the mean value of three identical dishes, and **error bars** are the upper 95% confidence intervals. *, $P<.001$, compared with the increase in H460/V1 cells. **D**) Overexpression of FADDm and celecoxib-induced DNA fragmentation. Cell lines were cultured in 96-well plates and treated on the second day with celecoxib as indicated. After 24 hours, DNA fragmentation was measured with the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals). Data are the mean value of three identical wells, and **error bars** are the upper 95% confidence intervals. *, $P<.001$ compared with the value in H460/V1 cells. **E**) Overexpression of FADDm and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Cells were treated with TRAIL at 25 ng/mL for 24 hours, and then DNA fragmentation was measured by use of the Cell Death Detection ELISA kit. Data are the mean value of three identical wells, and **error bars** are the upper 95% confidence intervals. *, $P = .002$, compared with that of H460/V1 cell lines. **F**) Overexpression of FADDm and celecoxib-induced caspase activation. Cells, as indicated, were treated with 50 μ*M* celecoxib for 20 hours, whole cell protein lysates were then prepared, and cleaved caspase 8 (Casp-8), caspase 9 (Casp-9), caspase 3 (Casp-3), and poly(ADP-ribose) polymerase (PARP) were detected by western blot analysis. Actin expression was used as a loading control. CF = cleaved form. All statistical tests were two sided.

compared with that in the corresponding untreated cells. After treatment with 75 μ*M* celecoxib, the amount of DNA fragmentation in both cell lines was further increased, but the increase in DNA fragmentation was statistically significantly lower in H460/Fm6 cells than in H460/V1 cells ($P<.001$) (Fig. 6, D). As a control, the amount of DNA fragmentation was statistically significantly lower in TRAIL-treated H460/Fm6 cells than in TRAIL-treated H460/V1 cells ($P = .002$) (Fig. 6, E). Clearly, FADDm overexpression suppressed celecoxib-induced apoptosis.

Furthermore, we found that celecoxib consistently induced a much higher level of cleavage of caspases 9, 8, and 3 and of PARP in H460/V1 cells than in H460/Fm6 cells (Fig. 6, F), demonstrating that FADDm overexpression also suppressed celecoxib's ability to activate the caspase 8–mediated caspase cascade. Thus, overexpression of FADDm appeared to suppress celecoxib-induced apoptosis, indicating that increased expression of death receptors, particularly expression of DR5, contributes to celecoxib-induced apoptosis, at least in human NSCLC cells.

To further explore the relationship between DR5 expression and celecoxib-induced apoptosis, we used siRNA methodology to silence the DR5 gene. DR5 siRNA transfection decreased both the basal level of DR5 expression and the level of celecoxib-increased DR5 expression and decreased the celecoxib-increased levels of cleaved caspase 8 and PARP (Fig. 7, A). Transfection with control siRNA did not interfere with celecoxib-induced increased DR5 expression or activation of

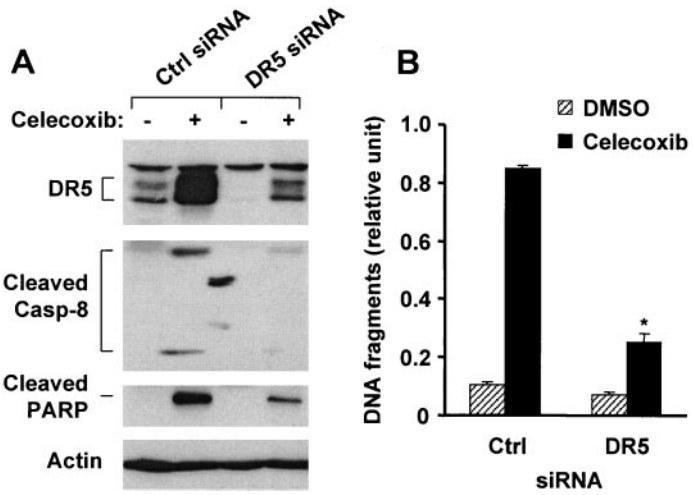

Fig. 7. Silencing of DR5 expression with small interfering RNA (siRNA) and celecoxib-induced caspase activation (**A**) and DNA fragmentation (**B**). H1792 cells were cultured in a 24-well plate and on the second day transfected twice with control (Ctrl) or DR5 siRNA with a 48-hour interval in between transfections. Forty hours after the second transfection, cells were treated with dimethyl sulfoxide (DMSO) or 50 μM celecoxib for 24 hours. **A**) DR5 expression and cleavage of caspase 8 (Casp-8) and poly(ADP-ribose) polymerase (PARP) were assessed by western blot analysis. The two sports between lanes 2 and 3 are nonspecific background material. **B**) DNA fragmentation was evaluated with the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals). Data are the mean value of three identical wells or treatments, and **error bars** are the upper 95% confidence intervals. *, $P<.001$ compared with that of control (Ctrl) siRNA-transfected cells treated with celecoxib. All statistical tests were two-sided.

caspase 8 and PARP cleavage (Fig. 7, A). In addition, celecoxib-induced DNA fragmentation was statistically significantly lower in DR5 siRNA–transfected cells than in control siRNA–transfected cells ($P<.001$) (Fig. 7, B). When treated with celecoxib, the level of DNA fragments (arbitrary unit) increased from 0.107 U (95% CI = 0.018 U to 0.197 U) to 0.848 U (95% CI = 0.814 U to 0.882 U) in control siRNA–transfected cells, whereas it increased only from 0.070 U (95% CI = 0.045 U to 0.097 U) to 0.249 U (95% CI = 0.127 U to 0.327 U) in DR5 siRNA–transfected cells. Thus, DR5 appeared to play an important role in mediating celecoxib-induced apoptosis.

Celecoxib, TRAIL, and the Induction of Apoptosis

Agents that induce death receptor expression usually enhance TRAIL-induced apoptosis. To determine whether celecoxib enhances TRAIL-induced apoptosis in NSCLC cells, we treated cells with TRAIL and various concentrations of celecoxib and assessed apoptosis. Treatment with TRAIL alone decreased survival of H358, A549, and H460 cells, and cell survival was further decreased by treatment with TRAIL in the presence of different concentrations of celecoxib (Fig. 8, A). In A549 cells, for example, celecoxib alone at 50 μM decreased cell survival by 38.7% (95% CI = 35.2% to 42.2%), TRAIL alone decreased cell survival by 29.3% (95% CI = 25.1% to 33.6%), but a combination of the two decreased cell survival by 77.5% (95% CI = 74.5% to 79.5%), which is greater than the sum of the effects of each agent alone. Similar results were observed with H358 and H460 cell lines. In addition, when DNA fragmentation in H358 cells was directly measured with an ELISA (Fig. 8, B and C) or by the TUNEL method (Fig. 8, D), a greater than additive amount of DNA fragmentation was detected in cells treated with a combination of celecoxib and TRAIL than in cells treated with either agent alone. As assessed by an ELISA, for example, the levels of DNA fragments were 0.082 U (95% CI = 0.017 U to 0.147 U) in untreated cells, 0.153 U (95% CI = 0.074 U to 0.233 U) in cells treated with TRAIL alone at 100 ng/mL, 0.412 U (95% CI = 0.166 U to 0.659 U) in cells treated with 50 μM celecoxib alone, and 1.129 U (95% CI = 0.617 U to 1.641 U) in cells treated with the combination of celecoxib and TRAIL (Fig. 8, B). As assessed by the TUNEL assay, TRAIL alone at 100 ng/mL and celecoxib alone at 50 μM induced 32.4% and 3.62% of cells to undergo apoptosis, respectively, but the combination of celecoxib and TRAIL caused 58.5% of cells to undergo apoptosis (Fig. 8, D).

To determine whether the combination of celecoxib and TRAIL enhances caspase activation in a supra-additive fashion, we examined caspase activation and caspase substrate cleavage in H358 cells treated with celecoxib alone, TRAIL alone, and the combination of celecoxib and TRAIL. Celecoxib alone at 50 μM or TRAIL alone at 100 or 200 ng/mL activated low levels of caspases 9, 8, 7, 6, and 3, and apparently did not increase cleavage of caspase substrates, including Bid, PARP, lamin A/C, and DFF45. However, the combination of celecoxib at 50 μM and TRAIL at 100 ng/mL or 200 ng/mL resulted in clearly increased caspase activation, as shown by decreased levels of uncleaved forms and/or increased levels of cleaved forms and increased cleavage of their substrates (Fig. 8, E). TRAIL alone at 400 ng/mL activated these caspases and increased cleavage of their substrates (Fig. 8, E, lane 8); however, the addition of celecoxib further increased both activities (Fig. 8, E, lane 5). Thus, celecoxib appeared to cooperate with TRAIL to activate both caspase 8– and caspase 9–mediated caspase cascades.

Discussion

In this study, we provided several lines of evidences to demonstrate that the extrinsic death receptor apoptotic pathway, particularly that involving DR5, plays a critical role in mediating celecoxib-induced apoptosis in human NSCLC cells. First, celecoxib activated caspase 8, and silencing of caspase 8 by caspase 8 siRNA abrogated celecoxib-induced caspase activation and DNA fragmentation, indicating that celecoxib induces apoptosis in a caspase 8–dependent manner. Second, overexpression of a dominant negative FADDm suppressed the ability of celecoxib to decrease cell survival and to increase caspase activation and DNA fragmentation, indicating that celecoxib induces apoptosis through an extrinsic apoptotic pathway. Third, the expression of DR4 and particularly of DR5 were induced in cells treated with celecoxib, and the silencing of DR5 expression by DR5 siRNA transfection blocked caspase 8 activation and decreased cell sensitivity to celecoxib-induced apoptosis, indicating that DR5 induction contributes to celecoxib-induced caspase 8 activation and apoptosis. Fourth, overexpression of BCL2 did not inhibit celecoxib-induced apoptosis, suggesting a less important role for the intrinsic mitochondria-mediated apoptotic pathway than for the extrinsic pathway in celecoxib-induced apoptosis, at least in NSCLC cells.

We also found that other NSAIDs (including NS398, DUP697, SC-58125, sulindac sulfide, and SC-560) increased the expression of DR5 and/or DR4 in NSCLC cells to various levels. The ability of an NSAID to increase the expression of death receptors, particularly DR5, is associated with its ability to

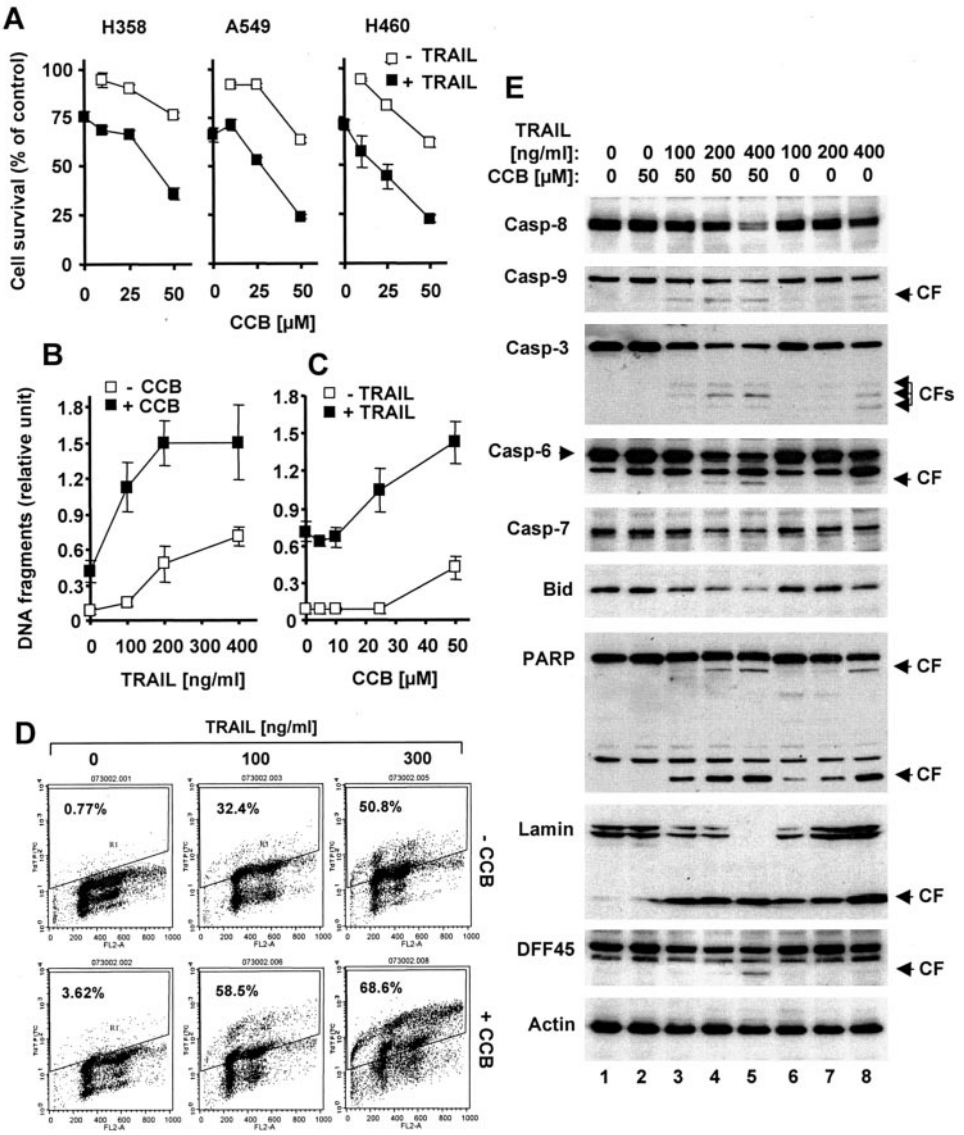

Fig. 8. Combination treatments with celecoxib and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and human non-small-cell lung carcinoma (NSCLC) cells. **A**) Cell survival. NSCLC cell lines, as indicated, were cultured in 96-well plates and treated on the second day with TRAIL alone at 400 ng/mL (H358), 300 ng/mL (A549), or 25 ng/mL (H460), celecoxib alone as indicated, or celecoxib plus TRAIL. After 20 hours, cell numbers were estimated by use of the sulforhodamine B assay, as described *(39).* Cell survival data are expressed as percent control dimethyl sulfoxide-treated cells. Data are the mean value of four identical wells, and **error bars** are the 95% confidence intervals. **B** and **C**) DNA fragmentation detected with an enzyme-linked immunosorbent assay (ELISA). H358 cells were cultured in 96-well plates and treated with 50 μ*M* celecoxib alone, TRAIL alone as indicated, or celecoxib plus TRAIL (**B**) or with celecoxib alone as indicated, TRAIL alone at 400 ng/mL, or celecoxib plus TRAIL (**C**). After 20 hours, cells were harvested, and DNA fragmentation was measured with the Cell Death Detection ELISA kit (Roche Molecular Biochemicals). Data are the mean value of three identical wells, and **error bars** are the 95% confidence intervals. **D**) DNA fragmentation detected with the terminal deoxynucleotidyltransferase-mediated UTP nick-end-labeling (TUNEL) assay. H358 cells were cultured in 10-cm dishes and treated on the second day with 50 μ*M* celecoxib alone, TRAIL alone at 100 or 300 ng/mL, or celecoxib plus TRAIL. After 18 hours, cells were harvested, and DNA fragmentation was measured with an APO-DIRECT TUNEL kit (Phoenix Flow Systems) by the manufacturer's protocol. The percent of apoptotic cells with DNA fragmentation is shown. **E**) Apoptosis detected by cleavage of caspases and their substrates. H358 cells were treated with 50 μ*M* celecoxib alone, TRAIL alone as indicated, or the combination of celecoxib and TRAIL for 16 hours. Whole cell protein lysates were then prepared from both detached (i.e., apoptotic death cells) and attached cells (i.e., cells that are undergoing apoptosis), and cleavage of caspases and their substrates was detected by western blot analysis. CCB = celecoxib; Casp = caspase; CF = cleaved form. Cleaved forms are indicated to the right. Caspase activation or cleavage of caspase substrates is indicated by a decreased level of the pro-form of the component and/or the appearance of cleaved forms.

induce apoptosis. Among the NSAIDs tested, celecoxib induced the highest expression of both DR5 and DR4. DUP697, which is structurally closest to celecoxib, was almost as active as celecoxib in inducing DR5 expression and decreasing cell survival; the level of DR4 expression induced by DUP697, however, was lower than that induced by celecoxib. Celecoxib increased the level of DR5 protein in all four cell lines tested but increased the level of DR4 protein in only two of the four lines (Figs. 2 and 5). Thus, the induction of DR5 expression may be more important than that of DR4 expression in mediating celecoxib-induced apoptosis. This result may explain the association between the ability of an NSAID to induce DR5 expression and its ability to induce apoptosis. At present, it is not clear whether induction of death receptor expression by an NSAID, such as celecoxib, is associated with its ability to inhibit cyclooxygenase 2. Because sulindac sulfide and celecoxib induce apoptosis independent of cyclooxygenase 2 levels and their induction of DR5 contributes to induction of apoptosis, the induction of death receptor expression by celecoxib and other NSAIDs may also be independent of their cyclooxygenase 2 inhibitory activity.

Celecoxib appears to increase the expression of DR5 by acting at the transcriptional level because the transcription inhibitor actinomycin D blocked the increased DR5 expression induced by celecoxib and because celecoxib did not alter DR5 mRNA stability. At present, the mechanism by which celecoxib increases DR5 expression is unclear. However, this effect is most likely p53–independent because celecoxib increased DR5 expression regardless of the p53 status of the cells examined. We showed that the protein synthesis inhibitor cycloheximide increased the level of DR5 mRNA and further enhanced the celecoxib-induced increase in DR5 mRNA expression. Although the mechanism of this effect is unclear, uncharacterized proteins may mask or inhibit the transcription of DR5. Cycloheximide may block the synthesis of these proteins and consequently block their suppression of DR5 expression.

Overexpression of BCL2 generally suppresses mitochondria-mediated apoptosis induced by many chemicals or small molecules *(24)*. In our study, we found that BCL2 overexpression did not suppress celecoxib-induced apoptosis, as has been reported in prostate cancer cells *(11),* and that celecoxib induced the same level of DR5 expression in both cells transfected with empty control vector and cells transfected with BCL2 vector. These results indirectly support a role of a death receptor–mediated extrinsic apoptotic pathway in celecoxib-induced apoptosis in NSCLC cells. Jendrossek et al. *(17)* have reported that Jurkat T cells overexpressing BCL2 were sensitive to celecoxib-induced apoptosis, which is consistent with our findings. However, in contrast to our results in NSCLC cells, they found that celecoxib could induce apoptosis in Jurkat T cells that lacked caspase 8 or FADD but did not induce apoptosis in the presence of a caspase 9 inhibitor and a dominant negative caspase 9 mutant. Thus, in Jurkat T cells, celecoxib appears to act through a caspase 9–mediated mitochondrial signaling pathway that leads to the induction of apoptosis independent of BCL2– and death receptor–mediated apoptotic pathways.

It should be noted that the concentrations of celecoxib used by Jendrossek et al. (75–100 μM) were higher than the concentrations used in our study (50 μM or lower). At such high concentrations, celecoxib might still induce apoptosis in cells deficient in caspase 8 or FADD if the intrinsic mitochondrial pathway could override the death receptor pathway. In our study, overexpression of the dominant negative FADDm only partially suppressed apoptosis when cells were treated by 75 μM celecoxib (Fig. 6, D). Celecoxib may also induce apoptosis by cell type–specific mechanisms because other studies have shown that BCL2 overexpression exerted different impacts on induction of apoptosis in different types of cancer cells. For example, overexpression of BCL2 failed to block TRAIL-induced apoptosis in Jurkat or myeloma cells *(44–46)* but suppressed TRAIL-induced apoptosis in human lung and prostate cancer cells *(37,47,48)*.

FADDm overexpresssion apparently protected cells from celecoxib-induced apoptotic cell death, as indicated by the increased number of floating or dead cells, the increased amount of DNA fragmentation, and increased level of caspase cleavage or activation in celecoxib-treated cultures compared with untreated cells (Fig. 6, C, D, and F). The FADDm overexpresssion apparently protected cells from celecoxib-induced apoptotic cell death as indicated by detecting the increase in floating or dead cells, increase in DNA fragmentation, and caspase cleavage or activation (Fig. 6, C, D, and F). However, the protective effect of the FADDm overexpression on cell number decrease caused by celecoxib was limited (close to 20% protection) (Fig. 6, B). If decrease of cell number is an outcome of the mixed effects due to growth arrest (i.e., proliferation inhibition) and apoptotic death, we expect only a partial rescue of cell number decrease, even by a substantial blockade of apoptotic death unless cell number decrease is caused purely by apoptosis. Thus, our data suggest that apoptosis only partially accounts for cell number decrease caused by celecoxib.

A potential limitation of our study is that the peak human plasma concentration of celecoxib after oral administration of a single dose of 400–800 mg ranges from 3 to 8 μM *(49,50),* which is considerably lower than the concentrations required to induce apoptosis in our study with human NSCLC cells and in other studies with different types of cancer cells. Currently, the tissue level of such a dose has not been determined. Nevertheless, the approach of celecoxib-based combination regimens for cancer chemoprevention and therapy and identification of celecoxib-derived novel anticancer drugs with more potent efficacy should be explored further.

Our findings that celecoxib increases the expression of death receptors and increases TRAIL-induced apoptosis indicate that combined treatment with celecoxib and TRAIL or celecoxib and agonistic anti-DR4 or -DR5 antibodies may be therapeutically useful in certain cancers. In this study, we have demonstrated that DR5 induction is a critical event in celecoxib-mediated apoptosis. Therefore, we suggest that induction of DR5 expression be explored further as a possible predictive biomarker for evaluating celecoxib and its derivatives as chemopreventive or therapeutic agents in clinical trials. Currently, several ongoing clinical trials are evaluating celecoxib alone or in combination with other agents for prevention or treatment of lung cancer *(51)*. Celecoxib is an FDA-approved and widely marketed drug that was originally developed as an anti-inflammatory drug, not as an anticancer drug. Celecoxib has a simple chemical structure and should be an ideal lead compound for developing novel derivatives with more potent apoptosis-inducing activity. In fact, celecoxib analogs have been developed, and the proapoptotic activity of some analogs is higher than that of celecoxib *(52,53)*. We suggest that death receptor induction be evaluated as a target for screening novel celecoxib-based anticancer drugs.

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